PHYTOCHEMICAL, PHARMACOLOGICAL ACTIVITY AND MOLECULAR DOCKING INVESTIGATIONS OF *Tetracera indica* (Christm. & Panz.) *Merr*., *Averrhoa bilimbi* Linn. AND *Gymnanthemum glaberrimum* (Welw. ex O.Hoffm.) H. Rob.

BY

ALHASSAN MUHAMMAD ALHASSAN

A thesis submitted in fulfillment of the requirement for the degree of Doctor of Philosophy in Pharmaceutical Sciences (Pharmaceutical Chemistry)

Kulliyyah of Pharmacy
International Islamic University Malaysia

APRIL 2018

ABSTRACT

Tetracera indica and *Averrhoa bilimbi* are used in traditional treatment of diabetes mellitus while *Gymnanthemum glaberrimum* is used as a remedy for skin cancer. The objectives of the present study were to investigate the pharmacological activity of leaves of *T. indica*, *G. glaberrimum* and *A. bilimbi*, and their phytochemical constituents. Ethanol extract from the leaves of *T. indica* was subjected to α-glucosidase inhibitory activity evaluation and isolation of bioactive compounds followed by molecular docking. Methanol extract of *G. glaberrium* leaves was subjected to cytotoxic investigations on A375, HT-29 and MCF7 cancer cell lines, followed by isolation of bioactive constituents and molecular docking. Purified compounds were characterized using spectroscopic analysis. Furthermore, the antioxidant activity of methanol extract of *A. bilimbi* leaves and its fractions was investigated and the active compounds were identified by LC-MS-QTOF analysis. Nine compounds were isolated from *T. indica* leaves which include; lupeol, betulinic acid, wogonin, norwogonin, quercetin, tectochrysin, kaempferol and two new sulphated flavones viz. 5,7-dihydroxyflavone-O-8-sulphate and 5-hydroxy-8-methoxyflavone-O-7-sulphate. Quercetin, kaempferol and 5,7-dihydroxyflavone-O-8-sulphate showed significant ($P<0.05$) α-glucosidase inhibitory activity with IC_{50} values of 61.86 ± 2.4, 68.46 ± 3.5 and 133.57 ± 5.2 μM respectively, compared to acarbose (IC_{50} 419.42 ± 20.29 μM). Molecular docking result showed favourable mode of interactions of these flavonoids in the active site of α-glucosidase. Furthermore, four compounds viz. nonacosanoic acid, lupeol, 5-methylcoumarin-4-β-glucoside and 4-hydroxy-5-methylcoumarin were isolated from *G. glaberrimum*. Lupeol displayed significant ($P<0.05$) antiproliferative effect against MCF-7 cell lines with IC_{50} of 34.15 ± 2.32 μg/mL. Lupeol and 5-methylcoumarin-4-β-glucoside displayed moderate cytotoxic effect on A375 cells with IC_{50} of 59.18 ± 2.70 and 91.84 ± 6.78 μg/mL. 4-Hydroxy-5-methylcoumarin, and lupeol displayed significant ($P<0.01$) cytotoxic effect on HT-29 cells with IC_{50} of 4.22 ± 0.13, 15.24 ± 1.15 μg/mL respectively, which is comparable with 5-fluorouracil (IC_{50} 8.00 ± 0.78 μg/mL). CDK2 receptor and CA IX and XII were identified through molecular docking as potential target for these compounds. The n-butanol fraction of *A. bilimbi* extract displayed significant ($P<0.05$) DPPH radical scavenging effect with IC_{50} (4.14 ± 0.21 μg/mL). It also exhibited significant ($P<0.05$) XO inhibitory effect with IC_{50} of 64.84 ± 3.93 μg/mL. Afzelechin 3-O-alpha-L-rhamnopyranoside and cucumerin A were identified through LC-MS-QTOF as possible metabolites responsible for the antioxidant activity of the n-butanol fraction.

خلاصة البحث

نباتات تيتراسيرا انديكا و ايفوريا بلمبي يستخدمان شعبيا لعلاج داء السكر أما نبات جمنازيم جلابيريم يتسخدم كعلاج لسرطان الجلد. أهداف الدراسة الحالية هي دراسة النشاط الدوائي لتلك النباتات ومكوناتهن الكيميائية. تم فحص فاعلية مستخلص الإيثانول من أوراق تيتراسيرا انديكا في تثبيط انزايم الالفا غليجوزيز وتم عزل المركبات الفعالة. في حين تم التحقق من سمية مستخلص الميثانول من أوراق جمنازيم جلابيريم باستخدام خطوط الخلايا السرطانيA375 ،HT-29وMCF7، تليها عزل المكونات النشطة بيولوجيا. تم وصف المركبات النقية باستخدام التحليل الطيفي. تم دراسة النشاط المضاد للأكسدة لمستخلص الميثانول من أوراق ايفوريا بلمبي ومشتقاتها وتم تحديد المركبات النشطة بواسطة تحليل LC-MS-QTOF. تم عزل تسعة مركبات من أوراق تيتراسيرا انديكا والتي تشمل لوبيول وحمض البوتيلينيك ،وجونين ، نوروجونين ، كيرسيتين ، تيكتوكيرسين ، كيمبيفرول بالاضافة الى اثنين من مركبات الفلافون الكبريتيية وهما 5،7–ديهيدروكسي فلافون–ثماني الكبريت و 5–هيدروكسي ميثوكسي فلافون–سباعي الكبريت .أظهر كيرسيتين و كيمبيفرول و 5،7–ديهيدروكسي فلافون–ثماني الكبريت نشاط تثبيط الالفا غلجوزيدز ($P < 0.05$) مع قيم IC_{50} من 61.86 ± 2.4 و 68.46 ± 3.5 و 133.57 ± 5.2 Mμ على التوالي ، مقارنة مع.اكاربوز (IC_{50} 419.42± 20.29 Mμ). أظهرت النتائج طريقة مواتية للتفاعلات بين هذه الفلافونيدات في الموقع النشط لـ الالفا غليجوزيز وعلاوة على ذلك ، تم عزل أربعة مركبات من نبات جمنازيم جلابيريم وهم حمض نوناكسانويك، لوبيول ، 5–ميثيل كومارين 4– بيتا جلوكوزايد و 4– هيدروكسي 5– ميثيل كومارين. أظهر لوبيول تأثير معتبر ($P < 0.05$) ضد خطوط الخلية MCF-7 مع IC_{50} من34.15 ± 2.32 ميكروغرام/مل.و كذلك أظهر كل من لوبيول و 5–ميثيل كومارين 4– بيتا جلوكوزايد تأثير متوسط ضد خلايا A375 مع IC_{50} من 59.18 ± 2.70 و 91.84 ± 6.78 ميكروجرام / مل. بالإضافة إلى ذلك فقد أظهر كل من لوبيول و 4– هيدروكسي 5– ميثيل كومارين تأثير ($P < 0.05$) سام للخلايا على HT-29 مع IC_{50} من 4.22 ± 0.13 ، 15.24 ± 1.15 ميكروغرام / مل على التوالي ، والتي يمكن مقارنتها مع 5–فلوروبوراسيل (ميكروجرام / مل 8.00 IC_{50}) . 0.78 ±كذلك تم تحديد مستقبلات CDK2 و CA IX و XII من خلال الالتحام الجزيئي كهدف محتمل لهذه المركبات. أظهر n- بيوتانول من مستخلص ايفوريا بلمبي تأثير جذري DPPH ($P < 0.05$) مع IC_{50} (4.14 ± 0.21 ميكروجرام / مل). كما أظهر تأثيرًا كبيرًا ($P < 0.05$) ولكن أقل من IC_{50}) XO 64.84 ± 3.93 µg / mL) .أخيرا تم تحديد كيومارين A وافزيليسين 3– الفا رامنو بيرانوزايد من خلال LC-MS-QTOF كنواتج ممكنة مسؤولة عن النشاط المضاد للأكسدة لمركب n–بيوتانول.

APPROVAL PAGE

The thesis of Alhassan Muhammad Alhassan has been approved by the following:

_____
Qamar Uddin Ahmed
Supervisor

_____
Alfi Khatib
Co-Supervisor

_____
Siti Zaiton Mat So'ad
Internal Examiner

_____
Jamia Azdina Jamal
External Examiner

_____
Zainul Amiruddin Zakaria
External Examiner

_____
Siti Aesah @ Naznin Muhammad
Chairman

DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Alhassan Muhammad Alhassan

Signature ..... Date .....

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH

PHYTOCHEMICAL, PHARMACOLOGICAL ACTIVITY AND MOLECULAR DOCKING INVESTIGATIONS OF *Tetracera indica* (Christm. & Panz.) Merr., *Averrhoa bilimbi* Linn. AND *Gymnanthemum glaberrimum* (Welw. ex O.Hoffm.) H. Rob.

I declare that the copyright holders of this thesis are jointly owned by Alhassan Muhammad Alhassan and IIUM.

No part of this unpublished research may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise without prior written permission of the copyright holder except as provided below

1. Any material contained in or derived from this unpublished research may be used by others in their writing with due acknowledgement.

2. IIUM or its library will have the right to make and transmit copies (print or electronic) for institutional and academic purposes.

3. The IIUM library will have the right to make, store in a retrieved system and supply copies of this unpublished research if requested by other universities and research libraries.

By signing this form, I acknowledged that I have read and understand the IIUM Intellectual Property Right and Commercialization policy.

Affirmed by Alhassan Muhammad Alhassan

……..…….. ……..
Signature Date

This thesis is dedicated to my father, Late Qadi Muhammad Alhassan who encouraged me to undertake this PhD and provided ample resources towards its accomplishment, but, as Allah so wished he did not survive to witness its completion. May Allah (SWT) have mercy on him and grant him Al-jannatul firdaus.

ACKNOWLEDGEMENTS

In the name of Allah, the Most Gracious, the Most Merciful. All praises and adoration are due to Allah alone (SWT) by whose mercy this work was accomplished. May His peace and blessings be upon Prophet Muhammad (SAW), his household, companions and all those that follow them with sincerity and piety.

I wish to express my sincere gratitude to my supervisor Assoc. Prof. Dr. Qamar Uddin Ahmed for his guidance, continuous support, encouragement and leadership, and for that, I will be forever grateful.

I am grateful to members of my supervisory committee and all those who provided their time, effort and support for this project.

I wish to express my profound appreciation to my dear mother; Hajiya Zainab, step mother; Hajiya Hadiza, wife; Hauwa, children; Khadija and Fatima, brothers; Aliyu, Aminu, Lukman and sisters; Hajiya Hadiza and Hajiya Rakiya. Your love, patience and support have been very wonderful and were vital to the successful achievement of this goal.

I also wish to express my gratitude to Usmanu Danfodiyo University Sokoto, Nigeria, for giving me the fellowship opportunity to undertake this study under the Tertiary Education Fund (Tetfund), 2013/2014 Intervention.

Finally, I am extremely indebted to the Ministry of Higher Education (MOHE), Malaysia and the Research Management Center, IIUM for providing financial support through Fundamental Grant Research Scheme (FRGS 13-089-0330), and Research Initiative Grant Schemes (RIGS 16-294-0458), respectively to accomplish this work.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Ac	Acetone
A375 cells	Human malignant melanoma cells
^{13}C-NMR	^{13}Carbon nuclear magnetic resonance
C	Carbon
CC	Column chromatography
$CDCl_3$	Deuterated chloroform
COSY	Homonuclear Correlation Spectroscopy
$CHCl_3$	Chloroform
2D	Two dimensional
dd	Doublet of the doublet
dL	Deci litre
DCM	Dichloromethane
DMSO	Dimethyl sulphoxide
DPPH	2, 2-diphenyl-1-1-picrylhydrazyl radical
EtOH	Ethanol
EtOAc	Ethyl acetate
$FeCl_3$	Ferric chloride
Fig.	Figure
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
^{1}H-NMR	Proton nuclear magnetic resonance
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum correlation
HT-29 cells	Human caucasian colon adenocarcininoma cells
HMBC	Heteronuclear multiple bond correlation
Hz	Hertz
H_2SO_4	Sulphuric acid
IDDM	Insulin dependent diabetes mellitus
I_2	Iodine
J	Coupling constant
Kg	Kilogram
m/*z*	Mass-to-charge ratio
MeOD	Deuterated methanol
MeOH	Methanol
MCF-7 cells	Human breast adenocarcinoma cells
MS	Mass Spectrometry
m	Multiplet
Mg	Milligram
NMR	Nuclear magnetic resonance spectroscopy
NOESY	Nuclear overhauser enhancement spectroscopy
NIDDM	Non-insulin dependent diabetes mellitus
OGTT	Oral glucose tolerance test
prep.	Preparative
ppm	Parts per million

s	Singlet
t	Triplet
T:E:F	Toluene: Ethyl formate: Formic acid
TLC	Thin layer chromatography
UV	Ultraviolet
2D	Two dimensional

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND AND JUSTIFICATION

Diabetes mellitus and cancer are two major diseases with huge impact on health worldwide. They contribute significant percentage to the overall number of deaths associated with non-communicable diseases (WHO, 2016).

Diabetes mellitus is a complex metabolic disorder characterized by increased blood glucose level resulting from insulin inadequacy or defect in insulin action or both. It affects both the young and the old, and it occurs in all regions of the world (Jaacks et al., 2016; Zimmet, 2017). Most cases of diabetes mellitus are grouped under two main categories namely; type-1 and type-2 diabetes. Type-1 diabetes is also known as insulin-dependent diabetes mellitus (IDDM), in which the pancreatic β-cells do not produce insulin at all, mostly befalls in children and young adults. It accounts for 5-10% of diabetes (Maahs et al., 2010). Type-2 diabetes is also known as non-insulin dependent diabetes mellitus, in which the pancreatic β-cells fail to produce sufficient amount of insulin or the body lacks the ability to properly utilize insulin. It is considered the most common form of the disease accounting for 90-95% of total cases of diabetes worldwide (Maahs et al., 2010). Diabetes mellitus has become a major public health problem across the world and is considered a key contributor to the global burden of diseases and disabilities. Its prevalence has been increasing rapidly due to obesity and sedentary life styles (Malik et al., 2013). It is considered as one of the largest epidemics in human history (Zimmet, 2017). According to the International Diabetic Federation (IDF, 2015), about 415 million

people are affected by the disease worldwide and the number is set to further increase beyond 600 million by 2040. Type-2 diabetes mellitus is one of the major diseases affecting Malaysians. Its incidence and prevalence is growing steadily. The prevalence of type-2 diabetes among adults in Malaysia (aged ≥ 18 years) has increased from 8.3% in 1996 to 11.6% in 2006 and subsequently to 15.2% in 2011 (Wan Nazaimoon et al., 2013). The Malaysian National Health Morbidity Survey conducted recently in 2015, recorded an overall diabetes prevalence of 17.5% (aged ≥ 18 years) (NHMS, 2015). Although tremendous progress has been made in the area of oral hypoglycemic agents, there are still unmet clinical needs with respect to the prevention of type-2 diabetes in high risk individuals, control of blood glucose levels and risk of developing long term complications (Bennett et al., 2014). The major drawbacks of the conventional agents used in the treatment of type-2 diabetes mellitus are frequent decrease in efficacy overtime leading to inadequate glycemic control as well as drug related adverse effects (Bennett et al., 2014). Hence, there is an urgent need for more researches to discover newer and more effective drugs to overcome diabetes.

Cancer is another important disease that constitutes a major public health problem. Cancer is a life threatening and debilitating incurable malady characterized by the abnormal rapid proliferation of cells that invade and destroy other tissues. It accounts for high number of morbidity and mortality associated with non-communicable diseases worldwide. It is the second leading cause of death after cardiovascular disorders. The total number of people living with cancer is growing steadily due to increase in population as well as exposure to risk factors such as smoking, poor diet, physical inactivity, reproductive changes and environmental pollution (Torre et al., 2015). Recent report has shown that, there were

approximately 14 million new cases and 8.2 million cancer related deaths in 2012, and more than 60% of the world's total new annual cases occur in Africa, Asia and Central and South America (Torre et al., 2015). These regions account of 70% of the world's cancer deaths. There are different types of cancers depending on the tissue or organ affected. The most common among them include cancer of the brain, lungs, breast, colorectal, skin, cervix, bladder and prostate. Today, cancer is one of the leading causes of mortality in Malaysia. Recent report from Malaysian cancer registry shows that, a total number of 103,507 new cancer cases were diagnosed during 2007 to 2011. About 45.2% of cases were reported in males while females have 54.8%. The five most common cancers among Malaysian males were cancers of the colorectum, lung, nasopharynx, lymphoma and prostate while cancers of the breast, colorectal, cervix uterus, ovary and lung are the most common among females (Azizah et al., 2016).

Plants used in folklore medicine represent an important source of biologically active compounds that could help to meet our current need for better anti-diabetic and anticancer drugs. According to the World Health Organization (WHO), large percentage of people living in Africa and Asia use the traditional or complementary alternative medicine to help meet some of their main healthcare needs. People in Europe, Australia, and United States have been increasingly embracing the use of traditional herbal medications to complement orthodox medicine (Qu et al., 2014; Rounds et al., 2017). Cumulative evidence from research with medicinal plants has revealed that their bioactivity is not due to a single chemical entity but often results from synergistic effect of multiple constituents, which implies that the secondary metabolites in a plant extract that are derived from their diverse chemistry, can act on

different and multiple targets involved in the pathogenic process, to augment overall therapeutic efficacy (Yang et al., 2014).

In spite of the great development in synthetic and combinatorial chemistry techniques, natural products remain an important source of molecules for discovery of new drug entities. For example, detailed investigation of new drugs approved by the US Food and Drug Administration (FDA) between 1981 and 2010 has shown that 34% of those drugs that were based on small molecules were either natural products or their direct derivatives including; hypolipidemics, anticancer drugs and immune suppressants (Newman and Cragg, 2012). From around the 1940s to the end of 2014, of all the 175 small molecules approved for cancer therapy, 85 (49%) are either natural products or their semi-synthetic derivatives (Newman and Cragg, 2016). The above analysis emphasizes the importance of natural product as source of valuable drugs for treatment of diseases and enhancement of general well-being. The discovery of drug from a medicinal plant often starts from the knowledge of its application in folk medicine, which is then subjected to pharmacological investigation to ascertain the authenticity of the claim. The natural product is required as a pure and characterized compound for further pharmacological investigation. This is achieved through bioactivity guided isolation and characterization using spectroscopic techniques.

In view of the foregoing, this research was focussed on the phytochemical and pharmacological investigations of *Tetracera indica, Averrhoa bilimbi* and *Gymnanthemum glaberrimum* leaves extracts which are claimed to be useful in the traditional treatment of diabetes mellitus and cancer but their phytoconstituents have not been well studied.